

Pathotypic characterization of the Newcastle disease virus isolated from commercial poultry in northwest Iran

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Abstract: Despite vaccination, outbreaks of Newcastle disease in commercial poultry flocks in northwest Iran have been regularly reported in recent years. This research was proposed in order to characterize, through a molecular approach, the genetic nature of strains of Newcastle disease virus isolated from commercial poultry flocks in northwest Iran. Ten Newcastle disease virus isolates were obtained from various districts of northwest Iran in 2010. The isolates were screened with *F* gene-targeting reverse transcription polymerase chain reaction (RT-PCR). The amplified products of virulent strains were sequenced. The deduced amino acid sequence of the fusion protein cleavage site indicated the presence of motif ¹¹²R-R-Q-R-R↓F¹¹⁷, typical for velogenic strains of NDV. These results confirmed that virulent NDVs are circulating in the region, causing economic losses within the poultry industry.

Key words: Newcastle disease virus, northwest Iran, fusion protein, cleavage site, RT-PCR, sequencing

1. Introduction

Newcastle disease (ND) is a highly contagious and widespread disease of poultry caused by Newcastle disease virus (NDV), also known as avian paramyxovirus type 1 (APMV-1) (1). The virus belongs to the genus *Avulavirus* within the family *Paramyxoviridae* (2). NDV is an enveloped virus, with a single-stranded negative-sense RNA genome. The genome is approximately 15 kb in length and follows the “rule of 6” as a prerequisite for viral replication (3). The genome contains 6 genes in the order 3′-NP-P-M-F-HN-L-5′, encoding for 6 structural and nonstructural proteins including nucleoprotein, phosphoprotein, matrix protein, fusion protein, hemagglutinin-neuraminidase protein, and large RNA polymerase (4,5). Two additional proteins, V and W, are derived by inserting 1 or 2 guanines during the transcription of *P* gene mRNA (6). The two surface glycoproteins HN and F are responsible for binding to host cell sialic acid receptors and for the fusion of the viral envelope to the host cell membrane, respectively (3,7).

NDVs can be grouped based on virulence into 5 pathotypes. The viscerotropic velogenic NDV is highly pathogenic and causes intestinal infection with high mortality, whereas neurotropic velogenic NDV is

responsible for respiratory and nervous symptoms, also with high mortality. The mesogenic strains of NDV are of intermediate virulence and often cause acute respiratory and nervous symptoms but have relatively low mortality rates. The lentogenic strains of NDV cause mild respiratory or enteric infections. The host lives longer in the asymptomatic enteric form and it is an ideal environment for replication and shedding of the virus (5,8). The velogenic and mesogenic strains have been identified as the causative agent in ND outbreaks in many countries. The lentogenic strains have been used as live vaccines to control the disease; however, outbreaks of ND have been consistently reported in vaccinated poultry (9).

Reports about the disease in Iran show evidence for the existence of virulent strains (10–13). However, the genetic nature of NDVs in clinically diseased flocks in the northwest of the country is unknown. Therefore, this study was designed to characterize NDV strains isolated from different restrictions in northwest Iran. The virulence of NDVs was evaluated by molecular analyses of the nucleotide and deduced amino acid sequences of the *F* gene. The results of this investigation will provide a more detailed understanding of NDVs circulating in the region and could help prevent future outbreaks of ND.

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2. Materials and methods

2.1. Sample collection

To ascertain the genetic nature of circulating NDV strains in northwest Iran, samples were collected during an emergent phase of the disease in 2010. A unit sample of 4 randomly infected birds was euthanatized from each of 10 commercial poultry flocks having relatively high mortality rates and showing clinical signs of ND. Brain tissues were collected aseptically and were immediately transported in a cold chain to a virology laboratory at the Faculty of Veterinary Medicine at Urmia University in Urmia, Iran. The samples were stored at -20°C until examination. A brief history regarding age at infection, clinical symptoms, mortalities, and course of infection was recorded.

2.2. Virus isolation

Viruses were isolated from a pooled homogenate of brain tissues by inoculation into the allantoic cavity of 9–10-day specific-pathogen-free (SPF) embryonated chicken eggs (ECEs). Allantoic fluids were collected after 5 days' incubation at 37°C (14,15). Initial characterization of all viral isolates was accomplished by hemagglutination inhibition (HI) with NDV-specific polyclonal antisera (Razi Vaccine and Serum Research Institute, Iran). The test was conducted by the conventional microtiter method. Positive and negative controls were run simultaneously with the test samples to validate the test (15).

2.3. RNA isolation

Viral RNAs were extracted from infected allantoic fluid using a High Pure Viral RNA isolation kit (Roche, Germany) as described in the manufacturer's instructions.

2.4. Molecular pathogenicity assessment

The presence of virulent F protein in the NDV positive isolates in the HI test was evaluated by the reverse transcription polymerase chain reaction method (RT-PCR) using Kant et al. primers (16). Two different 1-step RT-PCR reactions, targeting the *F* gene, were performed

using primer pairs A+B and A+C as general primers and virulent pathotype-specific primers, respectively (16) (Table 1). The reaction mixtures were prepared with the final volume of 25 μL as indicated by the manufacturer (Roche, Germany). The 1-step RT-PCR protocol was set up as 45°C for 45 min followed by initial denaturation at 94°C for 2 min and 35 cycles of denaturation at 94°C for 1 min, primer annealing at 58°C (for primers A+B) or 53°C (for primers A+C) for 1 min, and final extension at 70°C for 7 min.

2.5. Agarose gel electrophoresis

Amplified products were separated by 1.5% agarose gel electrophoresis in 5- μL volumes. The samples were run at 100 V for 1 h in a 1X TAE buffer stained with ethidium bromide and were visualized using a UV transilluminator (Model DOC-008, EEC). A 100-bp DNA ladder (Fermentas, Germany) was used as a weight marker.

2.6. RT-PCR for *F* gene sequencing

For this purpose, RT-PCR was performed using a Titan 1-step RT-PCR kit (Roche, Germany) to generate a 1349-bp amplicon encompassing the FPCS (17) (Table 2). The RT-PCR reaction mixture for each sample consisted of 2 μL of dNTPs mix (10 mM), 1 μL of forward primer (10 pM), 1 μL of reverse primer (10 pM), 7 μL of purified template RNA, 1.25 μL of DTT solution, 0.25 μL of protector RNase inhibitor, 5 μL of 5X RT-PCR buffer, and 0.5 μL of M-MuLV reverse transcriptase enzyme mix (Vivantis, Malaysia) in a final volume of 25 μL . RT-PCR was performed according to the following protocol: 45°C for 45 min for c-DNA synthesis followed by initial denaturation at 94°C for 2 min, and 35 cycles of 94°C for 1 min, 60°C for 1 min and 15 s, 68°C for 2 min and 15 s, with a final elongation step of 7 min for 70°C . The RT-PCR products were run in 2% low-melting agarose gel at 100 V for 1 h. A 1000-bp DNA ladder (Fermentas, Germany) was used as a molecular mass marker.

Table 1. Sense, sequence, and location of used oligonucleotide primers (16).

Code	Sense	Sequence	Location on <i>F</i> gene
A	+	5'-TTGATGGCAGGCCTCFFGC-3'	141-159
B	-	5'-GGAGGATGTTGGCAGCATT-3'	503-485
C	-	5'-AGCGT(C/T)TCTGTCTCCT-3'	395-380

Table 2. Primers used for RT-PCR amplification of the *F* gene (17).

Code	Sequence
P1	5'-TCACTCTATCCGTAGGATAACAAGAGTCTG-3'
P2	5'-GATCTAGGGTATTATTCCCAAGCCA-3'

2.7. Sequencing of PCR products

The bands of expected size (1349 bp) were cut from the agarose gel using a High Pure PCR Product Purification kit (Roche, Germany) according to the manufacturer's instructions. Sequencing was performed by the MWG-Biotech Company (Germany). The *F* gene was sequenced at least twice from independent RNA preparations in order to ensure consensus of sequencing.

2.8. Analysis of nucleotide and deduced amino acid sequences

The nucleotide sequence editing, analysis, and prediction of amino acid sequences for the *F* gene were conducted using DNASIS MAX software (version 3.0). Nucleotide and deduced amino acid sequences of the *F* gene were aligned using BioEdit software (version 7.0.9.0).

3. Results

After their incubation in ECEs, the allantoic fluids were examined for NDV following embryo death. Candling was done at 4-h intervals to determine the time of embryo death. The mean death time (MDT) of the viruses varied from 43 to 57 h. It was observed that all of the viruses had hemagglutination activity. HA titers ranged between 16 and 512. Furthermore, all of the samples were inhibited by NDV-specific antisera in an HI test.

Following RNA extraction from allantoic fluids as viral sources, RT-PCR reactions were conducted. In the first step of RT-PCR, a 362-bp fragment of the *F* gene was obtained using primer pair A+B in all NDV strains (Figure 1). Using primer pair A+C, a 254-bp fragment of the *F* gene was detected only in virulent NDVs in the second

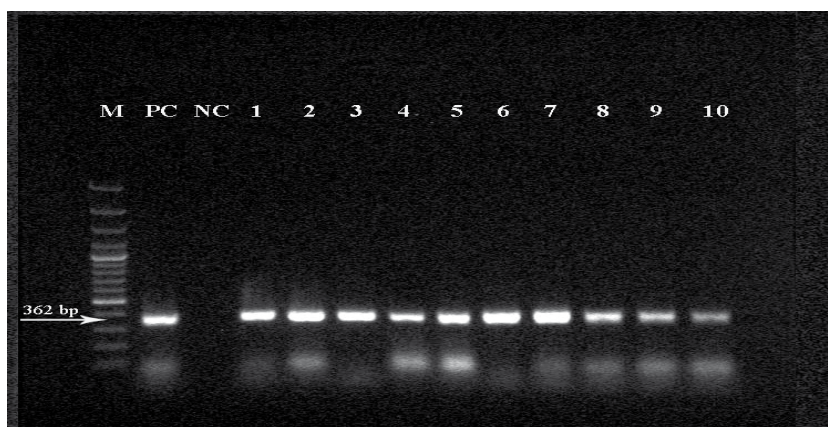


Figure 1. RT-PCR products (362 bp) generated from virulent and nonvirulent strains of NDV using primer pair A+B: M = 100 bp DNA Marker (Fermentas, Germany), PC= positive control, NC = negative control, Lanes 1-10 = field samples.

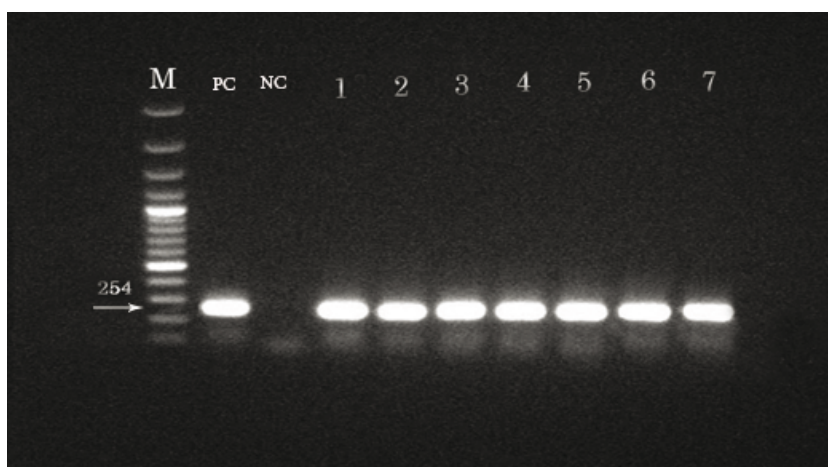


Figure 2. RT-PCR products (254 bp) generated from virulent isolates of NDV using primer pair A+C: M = 100 bp DNA Marker (Fermentas, Germany), PC = positive control, NC = negative control, Lanes 1-7 = field samples.

round of RT-PCR. Ten NDVs isolated from northwest Iran were determined to be virulent strains (Figure 2).

RT-PCR generated with virulent strains using primer pair P1+P2 resulted in a 1349 bp product, corresponding to nucleotides 335 to 1684 of the *F* gene. The amplicons were separated and sequenced, and the generated sequence data are available from GenBank, accession numbers KC161979.1–KC161988.1. From the pathotype prediction based on the FPCS, all of the isolates were placed in the velogenic group with the motif ¹¹²R-R-Q-R-R↓F¹¹⁷.

4. Discussion

Newcastle disease is one of the most serious poultry diseases due to its high rates of morbidity and mortality (8). During this study, isolates of NDV from infected commercial poultry flocks in different regions of northwest Iran were characterized pathotypically. All the field isolates of NDV were found to be virulent, with *F* gene-based RT-PCR, showing the FPCS amino acid sequence ¹¹²R-R-Q-R-R-F¹¹⁷.

The fusion protein is synthesized as the F0 precursor, becoming activated after post-translational cleavage into F1 and F2 fragments to initiate infection. The molecular determinant for NDV pathogenicity is the formation of active F1-F2 polypeptides by host proteases (9,18). According to the World Organization for Animal Health's molecular definition, virulent NDV has the amino acid sequence ¹¹²R/G/K-R-Q/K-K/R-R¹¹⁷ at the C-terminus of the F2 protein and phenylalanine (F) at residue 117 located at the N-terminus of the F1 protein, whereas ¹¹²G-R/K-Q-G-R-L¹¹⁷ is the consensus sequence for the cleavage site of low virulent viruses (19,20). Hence, pathotype prediction and diagnosis of the virulence of NDV can be performed using the FPCS sequence analysis.

Since the first RT-PCR described by Jestin and Jestin for NDV detection (21), developments in molecular biological diagnostic techniques have added to our increasing knowledge of the molecular basis of vNDVs. This has led many studies to investigate the possibility that conventional methods such as MDT and the intracerebral pathogenic index (ICPI) can be replaced by molecular-based techniques. Most molecular techniques involve a reverse transcription coupled to polymerase chain reaction. The generated PCR product can be used for nucleotide sequencing for cleavage site analysis and epidemiological studies (22–24).

ND is regarded as endemic in many countries and is often suspected in being involved in outbreaks in domesticated birds in the Middle East (10). Studies on NDV pathotyping in countries bordering Iran have also revealed the presence of virulent strains. In Turkey, virulent NDVs were isolated among the wild bird population in the Lake Van basin by analyzing for virulent F protein using real time RT-PCR (rRT-PCR) (25). Additionally, NDVs isolated from backyard poultry and wild birds were categorized by Dakman et al. as virulent based on their ICPI (26). ¹¹²RK/RQRR↓F¹¹⁷ and ¹¹²RRQKR↓F¹¹⁷ are the predominant motifs of FPCS in NDVs isolated from Pakistan during recent years (27,28). Our results are in agreement with previous studies reporting the detection of virulent NDVs in Iran (10,12). The FPCS of the collected isolates in this study are close to the FPCS of strains isolated previously from Iran (11) but different from those isolated from the 2010–2011 outbreaks in Shiraz, which possessed the FPCS motif ¹¹²R-R-Q-K-R-F¹¹⁶ (13). Overall, these results indicate that there are multiple velogenic strains circulating in Iran, causing outbreaks in poultry. Further, it is imperative to note the fact that samples were collected during a designated period throughout northwest Iran and that not all of the clinical outbreaks are necessarily included in the study, meaning that more diversity in vNDV strains in the region may exist. On the other hand, all of the infected farms were vaccinated against ND by dead and live attenuated vaccine strains. Therefore, the inability of vaccines to elicit protective immune responses against the prevailing field strains and to provide protection against field challenges needs to be evaluated. Thus, eradication of ND should focus on not only eliminating the presence of NDVs in flocks, but also increasing the efficacy of vaccinal strains (29,30).

We have provided evidence for the existence of vNDV in northwest Iran. Analysis of viral nucleotide distances, inferred phylogenetic relationships, and substitutions of deduced amino acid sequences of a greater number of field isolates from this part of the world would provide further information on pathotypic characteristics of prevalent ND viruses, which could be valuable in understanding the epidemiology of NDV and in developing better prophylactic measures.

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